

Comparative Studies on Tobacco Leaf

Use of a Rapid Extraction Technique

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A rapid extraction, disintegration technique was developed for tobacco leaf which was completed in 6 to 8 hours. Yields compared favorably with the usual Soxhlet procedures, and the method may be especially useful where prohibition of enzyme action is desirable. Exhaustive Soxhlet extraction demonstrated the presence of extractable material overlooked by the usual 20-25-hour procedure. This material is of interest since it is highly HBr-adsorbing and may include epoxides having significant tumor growth-regulating properties. Chloroform extractions gave yields comparable to exhaustive selective solvent (petroleum ether, ethyl ether, chloroform) extractions. Chloroform extracts were also reactive with hydrogen bromide.

Continued interest exists in the nature of substances in tobacco leaf which are soluble in various solvents. This report is concerned with the development of optimum yield techniques applicable to larger scale extractions with selected solvents. The materials obtained from the extraction of different type tobaccos are to be evaluated as carcinogenic agents and are for use especially in the identification of oxygenated compounds such as epoxides, peroxides, and hydroxy fatty acids. The possibility of the presence of epoxy compounds in tobacco is of particular interest since carcinogenic properties have been claimed^{3, 18, 42} for some epoxides. Long-chain epoxy-fatty acids have been reported present in over 40 species from 12 different

plant families.¹⁴ Also, oxygenated compounds having epoxide and α, β -unsaturated lactone functions, which possess tumor inhibitory activities, have been isolated from plants.²³⁻²⁵ Spiroepoxy compounds have been isolated from *Fusaria* and other *Hyphomycetes*;¹ some of these compounds have antifungal activity, and long-term feeding experiments are in progress to test for possible carcinogenic activity.¹

Previous reports^{11, 30, 34-39} from this Laboratory summarized the findings of extensive studies on the composition of the hexane-soluble material obtained from flue-cured tobacco leaf. A large-scale extraction was described³⁶ followed by a subsequent study on its higher fatty acid content.⁴⁰ Further work on tobacco leaf here included composition studies on the hydrocarbons,^{11, 34, 35, 37, 38} sterols,^{9-13, 35} phthalates,³¹ neophytadiene,^{2a} polyphenolic pigments,^{6, 8, 32} and solanesol-like substances.^{2b}

It was found expedient to affect rapid removal of petroleum-ether(PE)-soluble plant materials in previous in-

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vestigations^{18, 22, 28, 29} since highly active enzyme systems prevailed after grinding or crushing. Rapid extraction at room temperatures (or lower) prevented heat damage but did not affect yields^{18, 21, 22} of extractives, in comparison with those obtained by the usual Soxhlet procedures above room temperatures. These rapid extraction techniques were also found applicable in pilot-plant operations¹⁹ and now have been applied to tobacco leaf.

Methods

1. *Rapid Extraction Procedure*—As followed in this study it consisted in the use of a disintegrator (Waring Blendor*), equipped with a 1-liter stainless-steel assembly and an explosion-proof two-speed motor. The cutting blades could be driven at speeds up to 15,000 rpm. One-half of an intended 100.0-Gm. charge (about maximum size for this assembly) of tobacco leaf in 200 ml. of solvent was first disintegrated at low speed for about 30 seconds. The other 50.0 Gm. of tobacco and another 150–200 ml. of solvent were added and the disintegration continued for 2 minutes at low speed, followed at high speed for 1 minute. At high speed, the mixture's temperature rose to 35°C., when a doubly distilled fraction of petroleum ether (Skellysolve "F") was used as solvent (see below under *Soxhlet Procedure*). The finely disintegrated tobacco in solvent was then suction-filtered through a number S-1 (Republic Seitz Filter Corp., Newark, N. J.) filter pad. The tobacco marc was given two additional extractions by repeating the disintegration process with 250-ml. portions of fresh solvent.

* Mention of a commercial item does not imply endorsement by the Department over similar items not mentioned.

Finally, the marc was washed 3 times on the filter-plate with 100-ml. portions of solvent. All solutions were combined and the extractives were obtained after solvent removal in a rotating evaporator, under nitrogen.

2. *Soxhlet Procedure*—Tobacco used in these extractions was ground in a Wiley mill to pass a 200-mesh screen. Extraction thimbles (Whatman, 60 × 180 mm.) were used for 50- and 100-Gm. samples. The solvent-receiver flasks were of 1-liter capacity and condensers were protected from moisture with drying tubes (anhydrous calcium sulfate). Short thermometers were immersed in the tobacco during extractions to record temperature ranges. At the expiration of various time intervals, as indicated in the tables, the extracting solvents were removed and replaced with fresh solvents.

All petroleum ether was distilled twice, once in a 20-gallon stainless-steel evaporator, retaining a center cut which was then redistilled through a 1.5" × 18" glass column equipped with type 316 stainless-steel (0.24" × 0.24") protruded packing (Scientific Development Co., State College, Pa.); the center cut (PE, b.p. 35–50°C.) was retained for use in these investigations. The ether, methanol, and chloroform used were reagent grades.

3. *Analyses*—Moisture determinations⁵ were made on 5-Gm. samples, using the 3-hour oven drying technique, at 99.5 ± 0.5°C., followed by cooling in a desiccator.

Estimation of the quantity of epoxy compounds in a plant extract is usually achieved by a direct titration of the oxirane oxygen with hydrogen bromide in acetic acid, according to the Durbetaki⁷ hydrohalogenation procedure. This is the Official Tentative Method adopted by The American Oil Chemists' Society⁴ for the deter-

mination of a variety of epoxy compounds and epoxy resins. Carboxylic acids, aldehydes, ethers, esters, and peroxides do not interfere nor do hydroperoxides since they react too slowly with the reagent.^{7, 41} The Harris *et al.*¹⁵ modification of hydrohalogenation has been used in the present investigation to rule out the presence of cyclopropenoid compounds. In this modification,²⁷ epoxides titrate with the hydrogen bromide-acetic acid reagent at 3°C., whereas cyclopropenoid compounds titrate at 55°C. In recognition of the existence of the presence of substances present in plants which may react with hydrogen bromide and interfere with oxirane oxygen determinations,^{17, 26} the designation "hydrogen bromide consumption" or "hydrogen bromide equivalent" (HBE) has become one generally favored in preference to the oxirane oxygen terminology. HBE has been defined by Wilson *et al.*⁴³ as "the moles of hydrogen bromide consumed per mole of epoxyoleic acid (equivalent weight 296.5) times 100." This means arbitrarily assigning epoxyoleic acid, with a theoretical oxirane oxygen value of 5.3969%, an HBE value of 100. In the present work, 0.200–0.800-Gm. samples were titrated in duplicate and the average value reported. The values for duplicate samples did not deviate more than 1% from the average. It has recently been learned in this Laboratory that nicotine and related alkaloids absorb HBr. However, these compounds account for only a portion (about 5–50%) of the HBr absorbed by various solvent extracts of tobacco.

Results

In order to obtain an optimum figure for quantitative comparative purposes, a preliminary exhaustive extraction (Soxhlet) was made on

100.0-Gm. quantities of low moisture content (4.98%) flue-cured tobacco leaf with PE. Table 1 shows the yields of extractives obtained at various time intervals by prolonged extraction up to a total of 978.5 hours. The fractions obtained were dark-brown viscous liquids. The tobacco temperature during the extraction was consistently about 35°C. The first extract, removed after 0.5-hour operation, represented 20 exchanges of solvent through the tobacco. In addition to obtaining yield data, analysis was made for substances capable of hydrohalogenation. The material obtained from the 0.5-hour Soxhlet extraction possessed low hydrohalogenation properties. However, the material obtained by prolonged extraction, as illustrated by the three subsequent fractions, contained significant amounts of HBr-absorbing material. These higher HBE values compared favorably with those obtained for epoxidized linseed oil, 166.8, and for epoxidized soybean oil, 122.3. Published values²⁰ for these oils were mistakenly reversed. The HBE values also compared favorably with those for the natural seed oil, *Vernonia anthelmintica* (L.) Willd., 70.2;²⁰ this seed contains 25–30% oil of which 65–70% is a single compound, trivernolin, the triglyceride of 12, 13-epoxyoleic acid.^{18, 21} The literature values in these publications were expressed as oxirane oxygen percentages, but for comparative purposes here have been converted to an average HBE value.

To obtain larger quantities of PE extractives possessing high HBE values for composition studies, five 100.0-Gm. samples of flue-cured tobacco leaf were extracted for a prolonged period (528.5 hours). The average yields for the fractions, taken at various time intervals, are presented in Table 2. In the first extract, the yields varied from 4.50–5.06%. The general pattern

of HBr adsorption for these fractions followed that of the preliminary extractions. Further extractions with ethyl ether and with chloroform did not yield additional quantities of active materials (Table 2).

A comparison was made between the rapid disintegrator and the slower Soxhlet technique with respect to efficiency in PE extractions. Selected data are shown in Table 3. In initial experiments with the rapid procedure, 20 successive extractions were made on 50.0-Gm. samples of flue-cured tobacco leaf. No significant differences in yields were obtained when the sample size was increased to 100-Gm. (maximum capacity) quantities and the number of extractions reduced from 20 to 3. Also, there appeared to be no significant variance between extractions made at 5–10°C. and those made at 25–35°C. or between tobacco samples with moisture content of 5 and 15%. The rapid technique with PE gave yields comparable to those obtained with 25-hour Soxhlet procedures.

For comparative purposes, various tobacco types and mixtures were subjected to the rapid extraction techniques using PE; results are shown in Table 4.

In a comparison of the rapid extraction and Soxhlet methods it could not be assumed that temperature variation with higher boiling solvents would produce negligible yield differences as it did with PE (Table 3). Because of the high content of polar material in tobacco, methanol was selected as extracting solvent for further comparative purposes. Rapid extractions were made on flue-cured tobacco leaf with both cold (about 20°C.) and hot (about 60°C.) methanol. These were compared with Soxhlet extractions (temperature about 65°C.) and the results are presented in

Table 5. As expected, solvent at higher temperatures produced higher yields. However, comparable yields were obtained with the two methods as long as the extraction temperatures were close.

Because of special interest in Turkish tobacco, an aromatic type, extractions with chloroform were studied in further detail. Data in Table 6 are presented as an example of a different solvent application. By the rapid extraction technique the initial yield at 25–30°C. did not exceed 8.27% extractives. This figure was increased by only about 2% with two additional rapid extractions. However, further extraction by boiling the marc with chloroform followed by a 96-hour Soxhlet extraction gave an additional 2.37% (total figure 12.6% extractives). That this figure did not represent exhaustive extraction was shown by a 616-hour Soxhlet extraction of a 100.0-Gm sample with chloroform which gave a yield of 15.37% (HBE, 24.8).

Based upon the utility of these preliminary studies with chloroform a large-scale exhaustive extraction, using about 50 pounds of Turkish tobacco leaf, has been completed. Sufficient material has been obtained for extended composition studies with initial attention concentrated upon the HBr-absorbing material in the chloroform extract. Also, the marc from this chloroform extraction has received further attention for use in other extraction and composition studies. Results of these researches will be reported as progress is made.

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random checks using the Harris *et al.* modification of the hydrohalogenation procedure.

Table 1—Successive Soxhlet Extraction of a 100.0-Gm. Sample (4.98% Moisture) of Flue-cured Tobacco Leaf with PE

Fraction	Time Hr.	Yield			HBE
		Gm.	% ^a	% of Total	
1	0.5	4.70	4.94	57.7	5.2
2	96.0	1.80	1.89	22.1	121.0
3	240.0	1.01	1.09	12.8	202.0
4	642.0	0.60	0.63	7.4	136.0
Total	978.5	8.11	8.55	100.0	

^a All values are on a moisture-free basis (M.F.B.).

Table 2—Exhaustive Fractional Extraction of 100.0-Gm. Flue-cured Tobacco Leaf Samples (10.7% Moisture) with PE Followed by Ethyl Ether and by Chloroform.

Fraction	Time Hr.	PE ^a Yield		
		Gm.	% (M.F.B.)	% of Total
1	0.5	4.47	5.01	62.6
2	96.0	1.95	2.18	27.2
3	96.0	0.39	0.44	5.5
4	96.0	0.16	0.18	2.3
5	240.0	0.17	0.19	2.4
Total	528.5	7.14	8.00	100.0

		Ethyl Ether ^b Yield (after PE)		
		Gm.	%	% of Total
1	96.0	1.59	1.78	77.4
2	168.0	0.46	0.52	22.6
Total	264.0	2.05	2.30	100.0

		Chloroform ^b Yield (Following Ethyl Ether)		
		Gm.	%	% of Total
1	72.0	2.00	2.24	68.9
2	72.0	0.90	1.01	31.1
Total	144.0	2.90	3.25	100.0

^a Data are the averages of results from 5 extractions.

^b Average of two (100.0-Gm. samples) extractions.

Table 3—Comparison of Rapid Waring Blendor with Soxhlet Extraction Method, for Flue-cured Tobacco Leaf with PE

<i>Sample</i>		<i>Waring Blendor</i>		<i>Temp.</i> °C.	<i>Yield</i>	
		<i>Extraction</i>	<i>No. of</i>			
<i>Wt.</i> <i>Gm.</i>	<i>Moisture</i> %	<i>Time</i> <i>Min.</i>	<i>Extractions</i>		<i>Gm.</i>	<i>% (M.F.B.)</i>
50 ^a	5.09	3	20	5-10	3.54	7.46
50 ^a	5.09	3	20	25-35	3.70	7.80
100	15.0	3	3	25-35	6.20	7.30
100 ^a	5.09	3	3	25-35	7.33	7.72

		<i>Soxhlet</i>				
		<i>Hr.</i>				
40	5.57	25	1	35	3.06	8.10
40	5.57	25	1	35	2.90	7.75

^a Same tobacco air-dried from 15.0 to 5.09%.

Table 4—Rapid Extraction^a of 100-Gm Tobacco Samples with PE at 25–35°C.

<i>Tobacco Type</i>	<i>Moisture</i>	<i>Yield</i>	
	%	<i>Gm.</i>	<i>% (M.F.B.)</i>
Fire-cured leaf	7.21	5.78	6.23
Burley leaf	6.26	6.30	6.72
Turkish leaf	4.70	2.69	2.85 ^b
Cigar mixture	7.59	2.80	3.03
Flue-cured leaf	5.09	7.33	7.72
Maryland leaf	6.09	4.51	4.80
Cigarette mixture	5.30	4.20	4.44 ^c

^a One 400-ml., two 250-ml. extractions, and three 100-ml. washings of marc.

^b An additional 167-hour Soxhlet extraction raised this figure to 4.21%.

^c Raised to 6.15% by a 144-hour Soxhlet extraction.

Table 5—Comparison of Extraction Methods at Different Temperatures Using Methanol and Flue-cured Tobacco

<i>Temp., °C.</i>	<i>Rapid Method</i>		<i>Soxhlet Method</i>
	20	60	65 (24 hrs.)
<i>Yield % (M.F.B.)</i>	34.4	38.1	40.9

Table 6—Extraction of a 100.0-Gm. Sample of Turkish Tobacco Leaf (8.11% Moisture) with Chloroform

Extract No.	Technique	Temp. °C.	Yield		HBE
			Gm.	% (M.F.B.)	
1	Rapid ^a	25-35	7.60	8.27	4.04
2	Repeat 1	25-35	0.94	1.02	16.8
3	Repeat 1	25-35	0.92	1.01	19.8
4	Heat ^b	61	0.48	0.52	25.9
5	Soxhlet ^c	48-55	1.70	1.85	13.2
Total	—	—	11.64	12.67	—

^a One 400-ml., two 250-ml., 3-minute extractions and three 100-ml. washings (Waring Blender).

^b Boil marc 3 minutes with 250 ml. chloroform and wash 3 times.

^c Follow fraction 4 with 96-hour extraction.

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